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REVIEW

Fast-forward generation of effective artificial small RNAs for enhanced antiviral defense in plants

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Artificial small RNAs (sRNAs) are short ≈21-nt non-coding RNAs engineered to inactivate sequence complementary RNAs. In plants, they have been extensively used to silence cellular transcripts in gene function analyses and to target invading RNA viruses to induce resistance. Current artificial sRNA-based antiviral resistance in plants is mainly limited to a single virus, and is jeopardized by the emergence of mutations in the artificial sRNA target site or by the presence of co-infecting viruses. Hence, there is a need to further develop the artificial sRNA approach to generate more broad and durable antiviral resistance in plants. A recently developed toolbox allows for the time and cost-effective large-scale production of artificial sRNA constructs in plants. The toolbox includes the P-SAMS web tool for the automated design of artificial sRNAs, and a new generation of artificial microRNA and synthetic *trans*-acting small interfering RNA (syn-tasiRNA) vectors for direct cloning and high expression of artificial sRNAs. Here we describe how the simplicity and high-throughput capability of these new technologies should accelerate the study of artificial sRNA-based antiviral resistance in plants. In particular, we discuss the potential of the syn-tasiRNA approach as a promising strategy for developing more effective, durable and broad antiviral resistance in plants.

Keywords: small RNA; silencing, artificial microRNA; synthetic *trans*-acting small interfering RNA; plant virus, virus resistance

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Plant genomes encode diverse small RNAs (sRNAs) functioning in multiple silencing pathways [1]. MicroRNAs (miRNAs) and *trans*-acting small interfering RNAs (tasiRNAs) are two distinct classes of endogenous sRNAs that associate with an ARGONAUTE (AGO) protein to target and silence transcripts with highly complementary sequence. Silencing of targeted transcripts occurs through direct AGO-mediated endonucleolytic cleavage or through other cleavage-independent mechanisms [2]. Despite being functionally similar, miRNAs and tasiRNAs differ in their biogenesis pathways. MiRNA precursors are transcripts with imperfect self-complementary foldback structures processed by DICER-LIKE1 (DCL1), while tasiRNAs are produced in a more sophisticated manner. A miRNA/AGO complex cleaves a *TAS* transcript, RNA-DEPENDENT RNA POLYMERASE6 converts one of the cleavage products to double-stranded

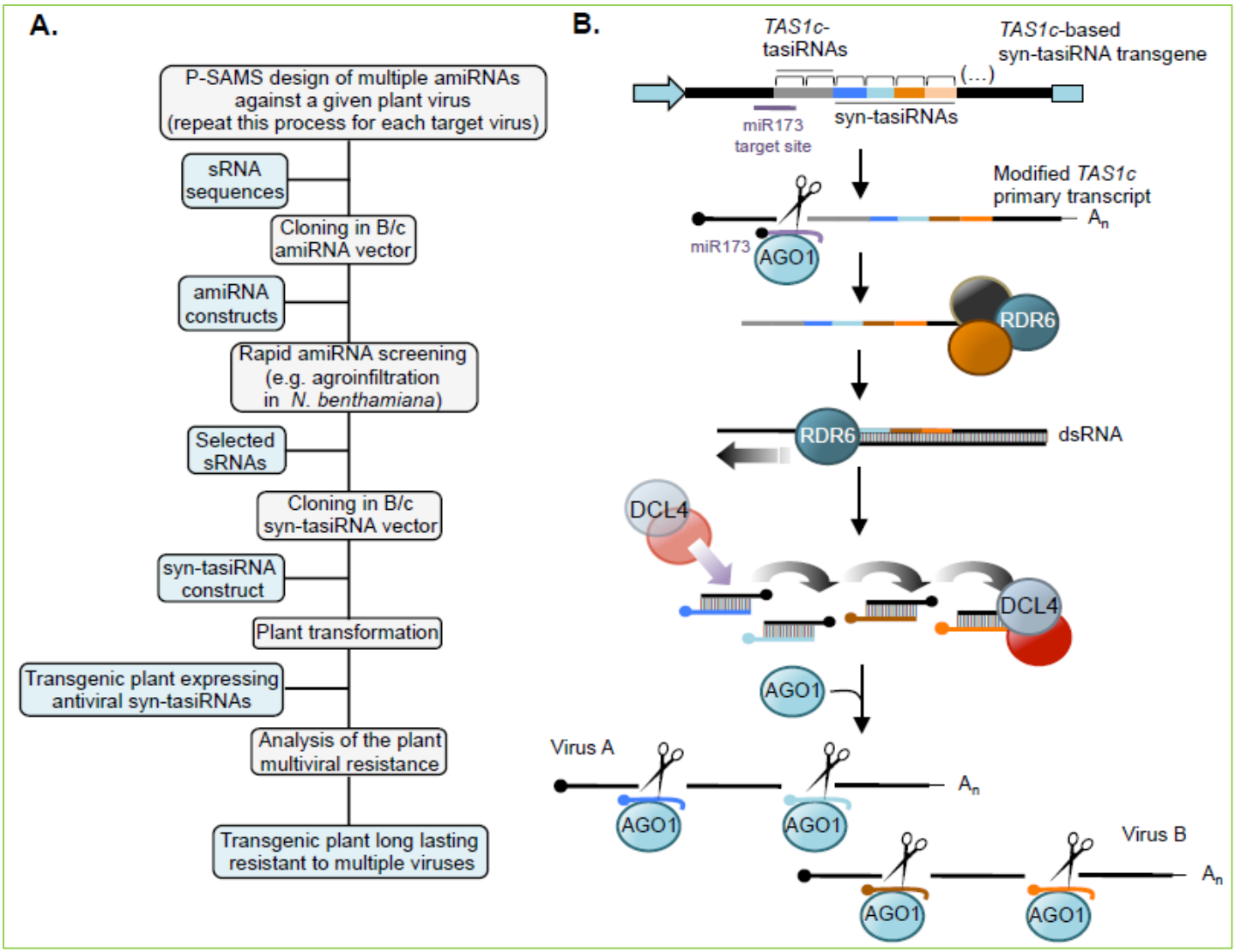

Figure 1. **Enhanced antiviral defense against multiple viruses by syn-tasiRNAs expressed from a single construct.** A, Diagram of the steps for the generation of effective and durable resistance against multiple viruses using new high-throughput artificial small RNA tools. Each step is described in light grey boxes. The product of each step is described in light blue boxes. B, *TAS1c*-B/c-based syn-tasiRNA pathway. AGO1/miR173 complex cleaves a modified *TAS1c* transcript including the syn-tasiRNA sequences. RDR6 complexes produce a dsRNA from the 3´ cleavage product, and DCL4 complexes process the dsRNA in several syn-tasiRNAs, each of which targets a different region within a particular virus. Multiple viruses may be targeted at different regions leading to effective, broad and durable resistance.

RNA (dsRNA), and DICER-LIKE4 (DCL4) sequentially processes the dsRNA into 21-nt tasiRNA duplexes in register with the miRNA-guided cleavage site [1, 2]. One strand of the miRNA or tasiRNA duplex is selectively incorporated to an AGO protein, usually AGO1.

Artificial miRNAs (amiRNAs) and synthetic tasiRNAs (syn-tasiRNAs) are designed to silence specific transcripts, and can be produced accurately *in planta* by expressing a functional miRNA or tasiRNA precursor with modified miRNA/miRNA* or tasiRNA sequences, respectively. Both classes of artificial sRNAs have been shown to inactivate selectively and effectively endogenous and reporter genes [3-7]. AmiRNAs have been also used to selectively confer antiviral resistance in transgenic plants [8]. However, this resistance is challenged by companion viruses in co-infected plants [9] and by virus sequence variants accumulating mutations in the amiRNA target-site [10, 11]. The co-expression of multiple artificial sRNAs targeting different target sites within a viral RNA or within multiple viral RNAs should result in a more effective, durable and broad antiviral resistance particularly in plant species infected by multiple related viruses. Indeed, the expression of multiple amiRNAs derived from different precursors or from a single polycistronic precursor and targeting different regions within a single viral RNA is effective [12-15], although the durability of the resistance has not been analyzed. Syn-tasiRNAs may also be an interesting source of antiviral resistance in plants as

analyzed in two recent reports, although with different conclusions possibly due to peculiarities of the constructs employed [16, 17].

Despite the extensive use of artificial sRNAs in plants, methods for designing and synthesizing artificial sRNA constructs have not been optimized for time and cost-effectiveness and high-throughput applicability. A platform has been recently developed, which includes molecular and bioinformatic tools for the simple and rapid design and generation of artificial sRNA constructs for highly specific and effective gene silencing in plants. Efficient methods were described to synthesize amiRNA and syn-tasiRNA constructs by directly ligating annealed DNA oligonucleotides containing the desired amiRNA or syn-tasiRNA(s) into a new generation of plant expression "B/c" vectors [18, 19]. B/c amiRNA vectors were validated in both eudicot and monocot species, and express a single amiRNA targeting one or multiple sequence-related transcripts [18, 19]. B/c syn-tasiRNA vectors were validated in *Arabidopsis thaliana* and allow the multiplexing of several syn-tasiRNAs to target different sequence-unrelated transcripts [18]. Additionally, the Plant Small RNA Maker Suite (P-SAMS, http://p-sams.carringtonlab.org), a wizard-assisted web-based tool for the simple and automated design of plant amiRNAs and syn-tasiRNAs, was developed [20]. P-SAMS outputs a list of suggested amiRNA or syn-tasiRNA together with the sequence of the two oligonucleotides needed for cloning the artificial sRNA into compatible B/c vectors. Several P-SAMS-designed amiRNAs aimed to target *Brachypodium distachyon* genes were validated in transgenic plants [18].

The rational use of these new tools should facilitate the generation of more effective and durable resistance against one or multiple sequence-unrelated plant viruses (Figure 1A). For example, P-SAMS can be used to design multiple amiRNAs against a particular virus. A module in P-SAMS is used to reduce the chances of off-targeting after selecting the plant species of interest. This process should be repeated for each virus to be targeted. P-SAMS-designed sRNA sequences can be directly cloned in B/c amiRNA vectors [18, 19]. AmiRNAs can be screened *in planta* to analyze their individual activity against their target virus. For most plant viruses this screening can be done quickly in agroinfiltration assays in *Nicotiana benthamiana* by co-expressing each amiRNA together with its target virus, and analyzing virus accumulation. The most effective amiRNA sequences for each virus can be selected and cloned in tandem in a B/c syn-tasiRNA vector [18]. Thus, syn-tasiRNAs targeting multiple sites per viral RNA can be expressed from a single construct in the plant species of interest to confer effective antiviral resistance against one or multiple viruses (Figure 1B). By targeting multiple sites per viral RNA, the antiviral resistance is expected to be effective and durable, as the possibility that the virus mutates all target sites to break the resistance appears unlikely. Efforts toward applying these new tools for enhanced antiviral resistance in plants are underway.

Conflicting interests

The authors have declared that no competing interests exist.

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